

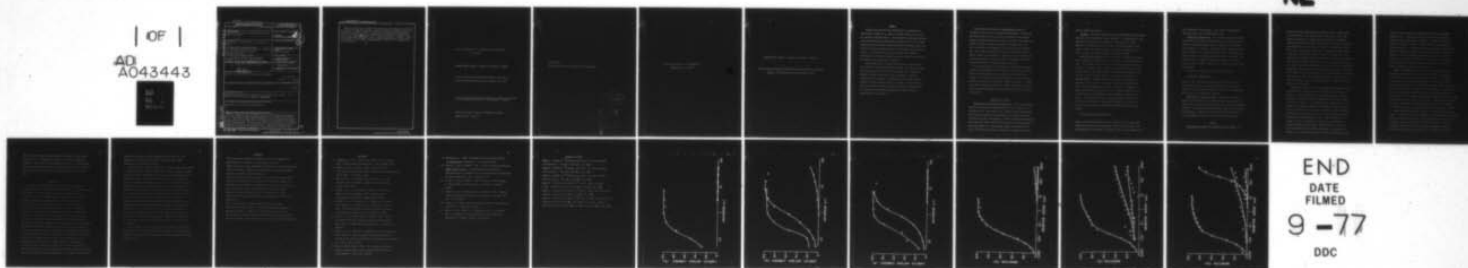
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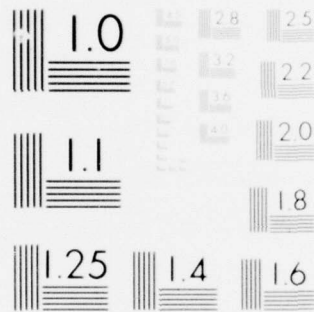
ARMY MEDICAL RESEARCH INST OF INFECTIOUS DISEASES FR--ETC F/G 6/15
ON THE CROSS-REACTIVITY OF STAPHYLOCOCCAL ENTEROTOXINS A, B, AN--ETC(U)
1977 L SPERO, B MORLOCK, J F METZGER

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From the United States Army Medical REsearch Institute of
Infectious Diseases, Fort Detrick, Frederick, MD 21701

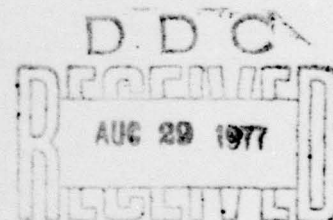
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On the Cross-Reactivity of Staphylococcal
Enterotoxins A, B, and C¹

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SUMMARY

Strong cross-reactions were demonstrated for staphylococcal enterotoxins B (SEB) and C₁ (SEC₁) by antigen-binding capacity and by competitive binding ability. Both SEB and SEC₁ combined completely with the heterologous antibody although requiring four times as much antiserum as the homologous enterotoxin and both displaced about one-third of the other enterotoxin from a heterologous antigen-antibody system. It is proposed that one of the three major antigenic determinants of these enterotoxins possesses a significant similarity but probably not an identity of structure.

SEB and SEC₁ did not combine with antiserum to enterotoxin A nor inhibit the reaction of SEA with anti-SEA. SEA had no intrinsic binding capacity for anti-SEB or anti-SEC₁ nor did it inhibit the binding of either enterotoxin to its own antibody. Affinity chromatography was employed to demonstrate that a small apparent binding of SEA to anti-SEB was due to antibody to SEA in the anti-SEB serum and that an almost complete displacement of SEC₁ binding to anti-SEC₁ was caused by contaminating SEC (about 0.01%) in preparations of enterotoxin A.

The enterotoxins produced by Staphylococcus aureus are identified on the basis of serological individuality. Of the five well-defined types A, B, C, D, and E, serological cross-reaction has been identified only between types A and E (1). The presence of common or similar antigenic determinants in enterotoxins A, B, and C has however been suggested by the work of Gruber and Wright (2). They reported in 1968 that both SEA² and SEC were bound by rabbit antibody to enterotoxin B. Johnson et al. (3) observed weak heterologous inhibition by SEA and SEC of the SEB-anti-SEB system and by SEB and SEC of the SEA-anti-SEA interaction.

We have investigated the interaction of SEA, SEB and SEC₁ with antisera to each enterotoxin by measurement of both antigen-binding capacity and the ability to bind competitively with homologous antigen. We report here that SEB and SEC₁ have a strong reciprocal reaction with each other's antibody. However, SEA does not combine with either anti-SEB or anti-SEC₁ or inhibit the binding of antigen in the two other homologous systems and neither SEB nor SEC₁ reacts with anti-SEA.

MATERIALS AND METHODS

Preparation and labeling of enterotoxins. The enterotoxins were produced and isolated by published methods (4,5) as modified in our laboratory (6,7). Each product gave a single line in sodium dodecyl sulfate polyacrylamide gel electrophoresis (8) and by all physical and biological tests was estimated to be more than 99% pure enterotoxin. The toxins were labeled with ¹²⁵I by the gaseous diffusion method of Gruber and Wright (2). One milligram samples were labeled with 1 mCi of ¹²⁵I with an efficiency of 10-15% to an extent of about one

atom of iodine per molecule.

Antisera. New Zealand white rabbits were immunized by intracutaneous injection of the enterotoxin without adjuvant³. A regimen based on that developed by Silverman (9) was employed. Only those sera giving identical Ouchterlony titers were pooled. The antisera used in this study contained the following levels of specific immunoglobulin: anti-SEA, 2.2 mg/ml; anti-SEB, 0.8 mg/ml; anti-SEC₁, 0.8 mg/ml.

Antigen-binding capacity. Tests for measuring binding to antibody were performed by a modification⁴ of the method of Jonsson and Kronvall (10). This technique is based on the ability of protein A-containing strains of S. aureus to react specifically and with high affinity with the Fc portion of IgG (11). Samples of labeled antigen (100 ng in 500 μ l) were added to 500 μ l of twofold serially diluted antiserum in phosphate-buffered saline containing 0.5% bovine serum albumin and incubated at 4°C for 18 hr. To separate bound from unbound antigen 100 μ l of a 10% suspension of the Cowan I strain of S. aureus was added, and the tubes were mixed on a vortex shaker, allowed to stand for 10-20 min at room temperature, and centrifuged at 1700 x G for 10 min. The supernatant fluid was decanted and the tubes drained. The tubes were counted in a gamma counter (Searle) for 1 min. The amount of antigen bound to antibody was calculated by the following formula:

$$\% \text{ Antigen combined} = \frac{A - C}{B - C} \times 100$$

where A is the radioactivity in the test sample, B is the total added radioactivity, and C is the nonspecific radioactivity occluded by the microorganism in the absence of antibody. Fifty per cent endpoints were

best determined by log antibody - log % antigen-combined plots which were linear in the 20-80% binding range.

Competitive binding assay. The volume of antiserum sufficient to bind 50% of the labeled antigen (100 ng) was added to varying amounts of unlabeled inhibitor and the solution was incubated at room temperature for 20 min. Labeled homologous antigen was added and the mixtures were incubated overnight at 4°C. The tubes were then processed as in the antigen-binding capacity technique. The delayed addition of labeled antigen increased the sensitivity of the assay about fourfold. Beyond 30 min no increase in sensitivity was observed.

The per cent inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{D - E}{D - C} \times 100$$

where D is the radioactivity in the absence of inhibitor, E is the radioactivity in the presence of inhibitor, and C is the radioactivity of the labeled antigen occluded by the precipitating microorganism in the absence of antibody.

Preparation of affinity columns. Proteins were coupled to Sepharose 4B (Pharmacia) by the method of Cuatrecasas *et al.* (12) except that the cyanogen bromide was added in a solution of dimethyl formamide. Antibody was precipitated three times by 1/3 saturation with $(\text{NH}_4)_2\text{SO}_4$. About 50 mg were coupled and the final volume of settled adsorbent was made to 10 ml with untreated Sepharose.

RESULTS

Antigen-binding capacity of heterologous enterotoxins. The

6

results of antigen-binding capacity determinations are shown in Fig. 1-3 for antisera to SEA, SEB, and SEC₁, respectively. All of the labeled enterotoxins are completely precipitable in a homologous system confirming that minimal alteration in the enterotoxin structure was brought about by this level of iodination. No binding of either SEB or SEC₁ to anti-SEA was detectable. Significant binding capacity of SEC₁ for anti-SEB (Fig. 2) and of SEB for anti-SEC₁ (Fig. 3) was apparent with essentially all of the labeled enterotoxins being removed from solution as immune complexes by the precipitating agent for the IgG. About four times more antiserum was required for each heterologous antigen over that required for the homologous interaction. The ratios of 50% endpoints were 4.2 for SEC₁/SEB with anti-SEB and 4.1 for SEB/SEC₁ with anti-SEC₁. These data indicate quite clearly that there is at least one antigenic region on SEB and SEC₁ with a significant degree of similarity of structure.

Enterotoxin A did not bind to anti-SEC₁ but an apparent interaction with anti-SEB to the extent of 17% was observed. This binding, if specific, is only 1/1000th that of the homologous toxin. Another possibility, however, was that the rabbit antiserum to SEB contains low levels of anti-SEA. It is known that the organism used for the production of SEB also elaborates small amounts of SEA (13) and the protocol employed for the production of antiserum involves multiple injections over a long period of time. To distinguish between these alternatives affinity columns were used. When anti-SEB was run through a column of SEA bound to Sepharose 4B, the effluent no longer combined with labeled SEA. Although within the limits of assay the antibody titer to SEB was unchanged, this experiment is subject to the criticism that a small fraction of anti-SEB molecules

could have been removed by the SEA-Sepharose without a detectable change in titer. Therefore, antiserum to SEB was passed over an affinity column of SEB bound to Sepharose. A column of untreated Sepharose was run in parallel to obviate dilution and nonspecific adsorption effects. As anticipated, antibody to SEB was quantitatively removed but 80% of the capacity to bind to SEA remained in the column effluent. Since the adsorbent had the capacity for at least 100 times as much anti-SEB as was added, it seems quite clear that what passed through the column was anti-SEA and therefore there is no intrinsic binding of SEA to antibody for SEB.

Competitive inhibition. The competitive binding or inhibition experiments are presented in Fig. 4-6 for the labeled enterotoxin-anti-enterotoxin systems A, B, and C₁ respectively. It is apparent that in each case the unlabeled homologous enterotoxin was capable of 100% inhibition, that is, of completely displacing labeled enterotoxin from specific antibody. SEC₁ did not compete with the SEA system at all and a very minor displacement by SEB is of questionable significance (vide supra) (Fig. 4). Enterotoxin B competed successfully with ¹²⁵I-SEC₁ for anti-SEC₁ and the converse was also true. A displacement of 24% was observed by SEB (Fig. 6) and of 42% by SEC₁ (Fig. 5). These effects were achieved, however, at enormous molar ratios of the order of 10⁵:1.

Only marginal inhibition was found for SEA against the SEB system (Fig. 5). However, there was an apparent, almost quantitative displacement by SEA of labeled SEC₁ (Fig. 6). This surprising observation was also found with another preparation of SEA and another batch of rabbit anti-SEC₁. The shape of the curve, however, suggested an impurity of SEC in the enterotoxin A preparation. Accordingly, a solution of

SEA was passed through an affinity column of antibody to SEC₁ coupled to Sepharose 4B. The inhibitory activity of the SEA was completely eliminated. This result is also presented in Fig. 6. To our knowledge this is the first report that the organism producing SEA also synthesizes SEC. The level of contamination in the SEA preparation is less than 0.01%.

DISCUSSION

The lack of serological cross-reaction among the staphylococcal enterotoxins does not, of course, preclude the presence of common antigenic determinants. The present data demonstrate convincingly that this situation does exist for SEB and SEC₁. The theory of matrix formation and the experimental observation that SEB and SEC₁ do not form a precipitate with each other's antiserum limit the possible number of common, simultaneously reacting determinants to two. A simple but obviously not unique model which appears to fit the data involves but a single common determinant. It is proposed that this epitope is one of three major antigenic regions⁵ and gives rise to about one-third of the antibody populations in antisera to SEB and SEC₁. If the heterologous determinant then possessed an equivalent affinity constant a threefold excess of the amount of antibody observed for the binding of the homologous enterotoxin would be required. The fourfold ratio obtained is very close to this, but it perhaps suggests either a lower concentration of the particular antibody molecule or a reduced affinity. The apparent levelling off of the competitive binding curves at 30-40% inhibition is consistent with this picture. An inhibition of 60-70% would be anticipated with two determinants of equal immunogenicity. The shape of these curves

would seem to indicate a reduced affinity and therefore that some difference in structure exists between the functionally similar determinant in enterotoxins B and C₁.

We have found no evidence of interaction of SEB or SEC₁ with anti-SEA or of SEA with either anti-SEB or anti-SEC₁. The small apparent binding capacity of SEA for anti-SEB [also observed by Gruber and Wright (2)] is demonstrably due to the presence of antibody to SEA in the antiserum to SEB. Our failure to find heterologous inhibition of the enterotoxin B-anti-SEB system by SEA and of the enterotoxin A-anti-SEA system by SEB or SEC₁ is in disagreement with the observations of Johnson et al. (3). While their assay system is considerably more sensitive than ours, we were able to discern no effect at molar ratios of inhibitor to homologous antigen that were equivalent or greater than their ratio for 33% inhibition. Duplicating the incubation conditions Johnson et al. employed did not alter our results. It is of great significance, we feel, that no binding was observed for these heterologous reactions in the assay for antigen-binding capacity. Combination in the absence of a homologous antigen is surely a more sensitive indicator than in its presence.

We are studying the cross-reaction of the polypeptides of SEB and SEC₁ formed by limited digestion with trypsin (6,7). This should permit a better understanding of the location of the common antigenic determinant described here.

FOOTNOTES

¹The views of the author do not purport to reflect the positions of the Department of the Army or the Department of Defense.

²Abbreviations used: SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; SEC, staphylococcal enterotoxin C, (SEC₁ is the variant with the more alkaline isoelectric point).

³In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

⁴J. F. Metzger and A. D. Johnson, manuscript in preparation.

⁵The effective antigenic valence of SEB as determined by quantitative precipitin analysis is three (14). For SEA and SEC₁ values of 3-4 were obtained (L. Spero, unpublished observations) and for the purposes of this discussion a value of three is assumed for all of the enterotoxins.

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LEGEND FOR FIGURES

Figure 1. Binding of ^{125}I -labeled enterotoxins to rabbit antiserum to enterotoxin A. SEA (Δ); SEB (\bigcirc); SEC₁ (\square).

Figure 2. Binding of ^{125}I -labeled enterotoxins to rabbit antiserum to enterotoxin B. SEA (Δ); SEB (\bigcirc); SEC₁ (\square).

Figure 3. Binding of ^{125}I -labeled enterotoxins to rabbit antiserum to enterotoxin C₁. SEA (Δ); SEB (\bigcirc); SEC₁ (\square).

Figure 4. Inhibition of binding of ^{125}I -SEA to rabbit antiserum to SEA. Inhibitors used were: SEA (Δ); SEB (\bigcirc); SEC₁ (\square).

Figure 5. Inhibition of binding of ^{125}I -SEB to rabbit antiserum to SEB. Inhibitors used were: SEA (Δ); SEB (\bigcirc); SEC₁ (\square).

Figure 6. Inhibition of binding of ^{125}I -SEC₁ to rabbit antiserum to SEC₁. Inhibitors used were: SEA (\blacktriangle); SEA (Δ), after passage through an affinity column of anti-SEC₁ bound to Sepharose 4B; SEB (\bigcirc); SEC₁ (\square).

Fig. 1

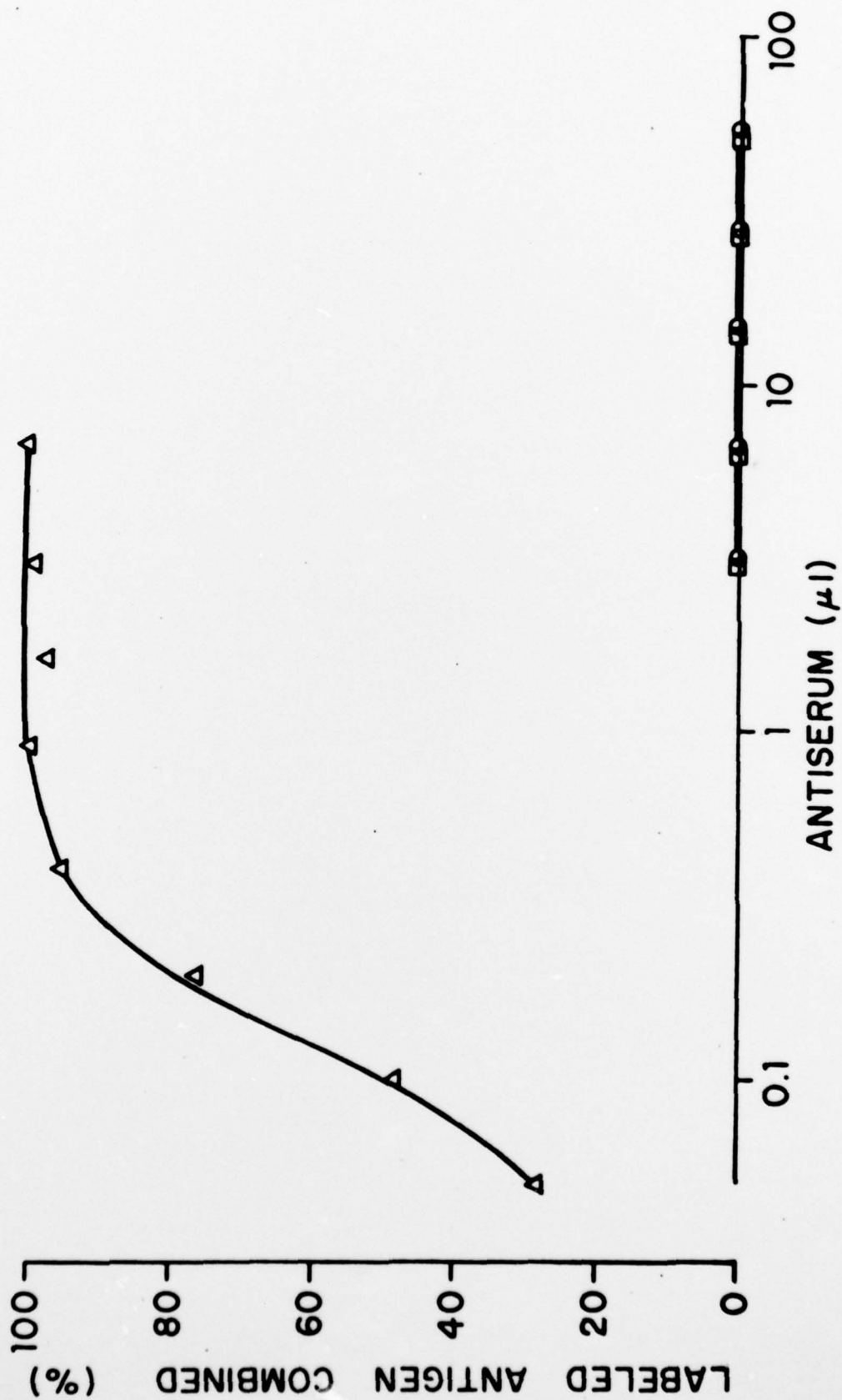


Fig. 2

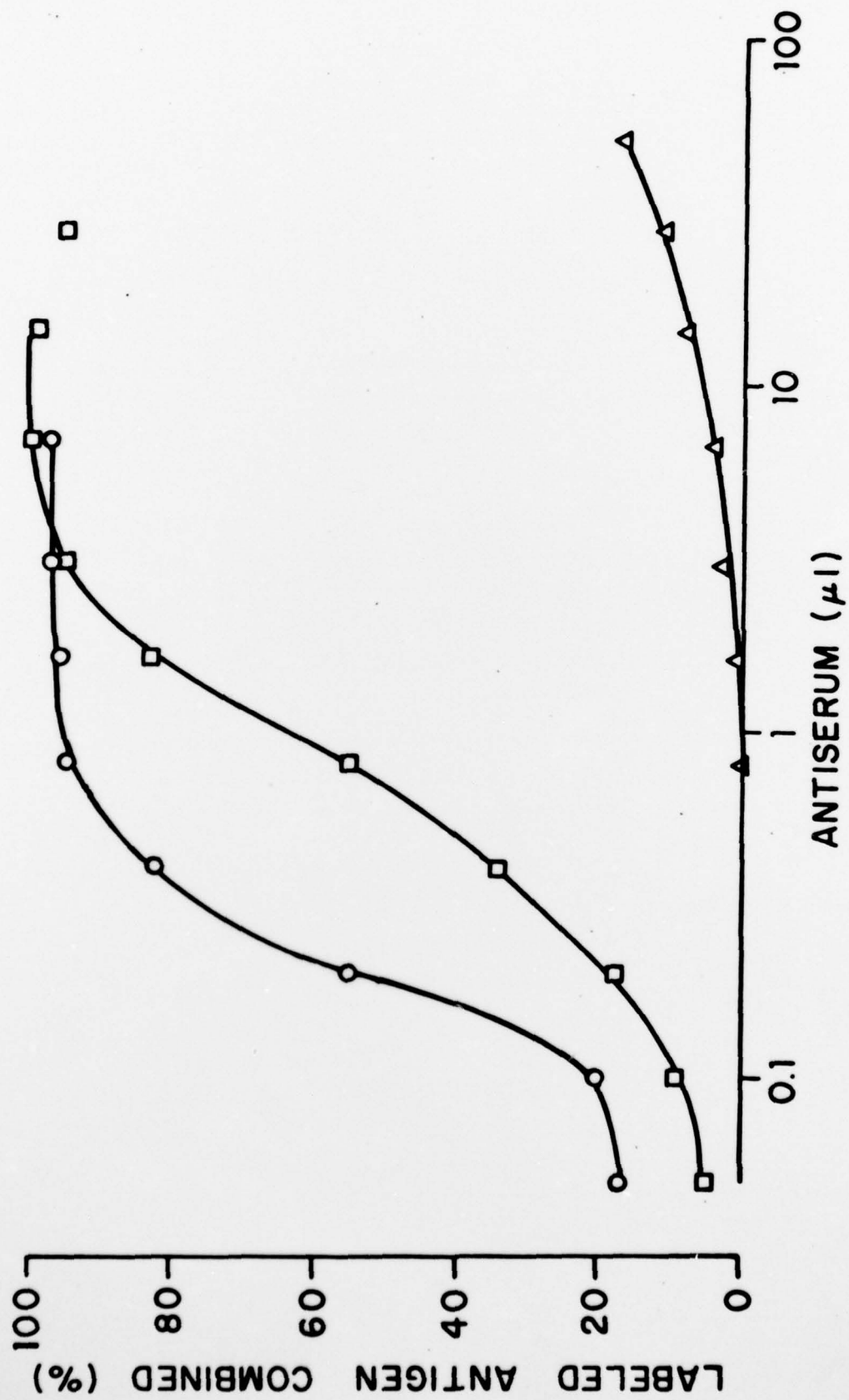


Fig. 3

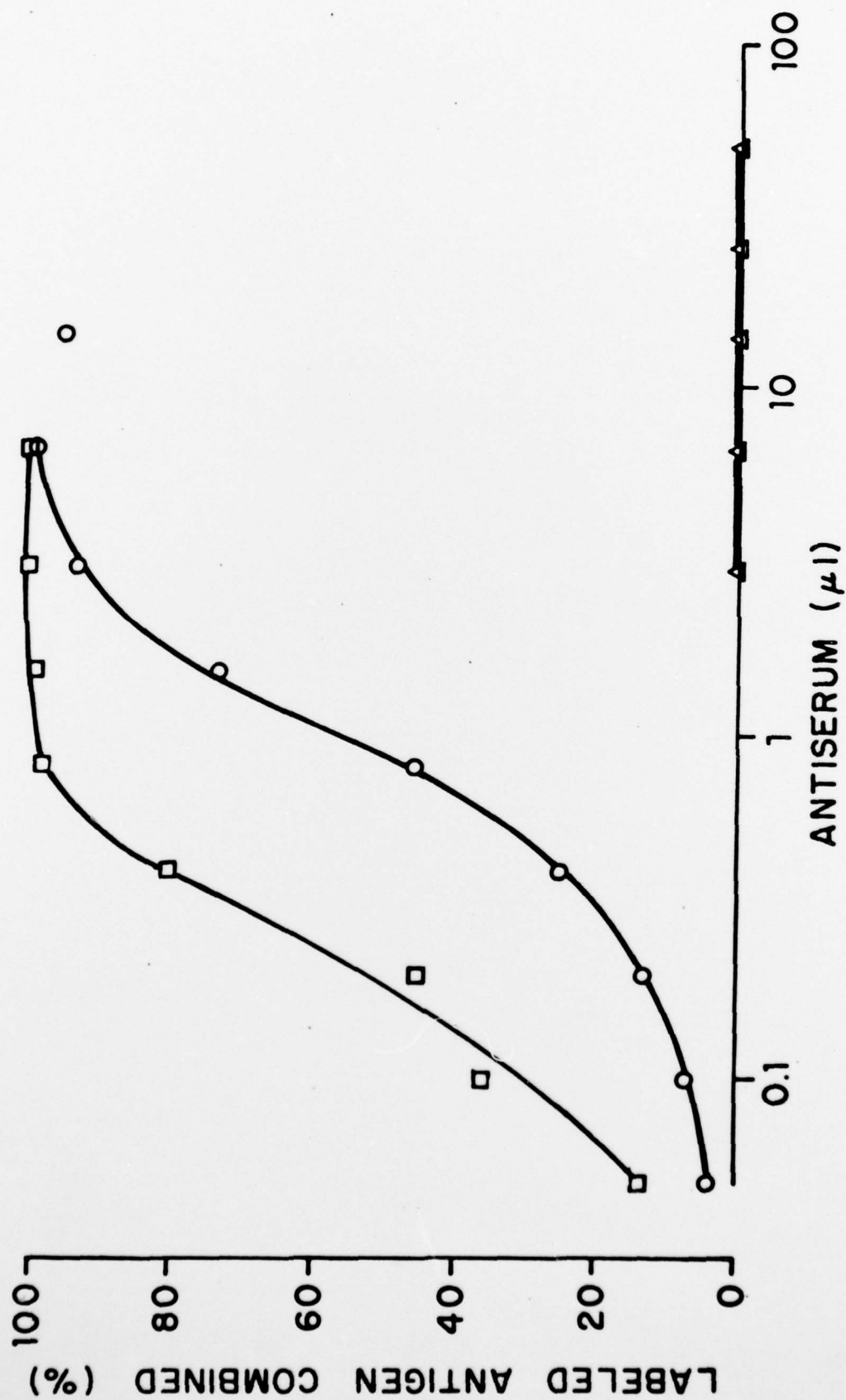


Fig. 4

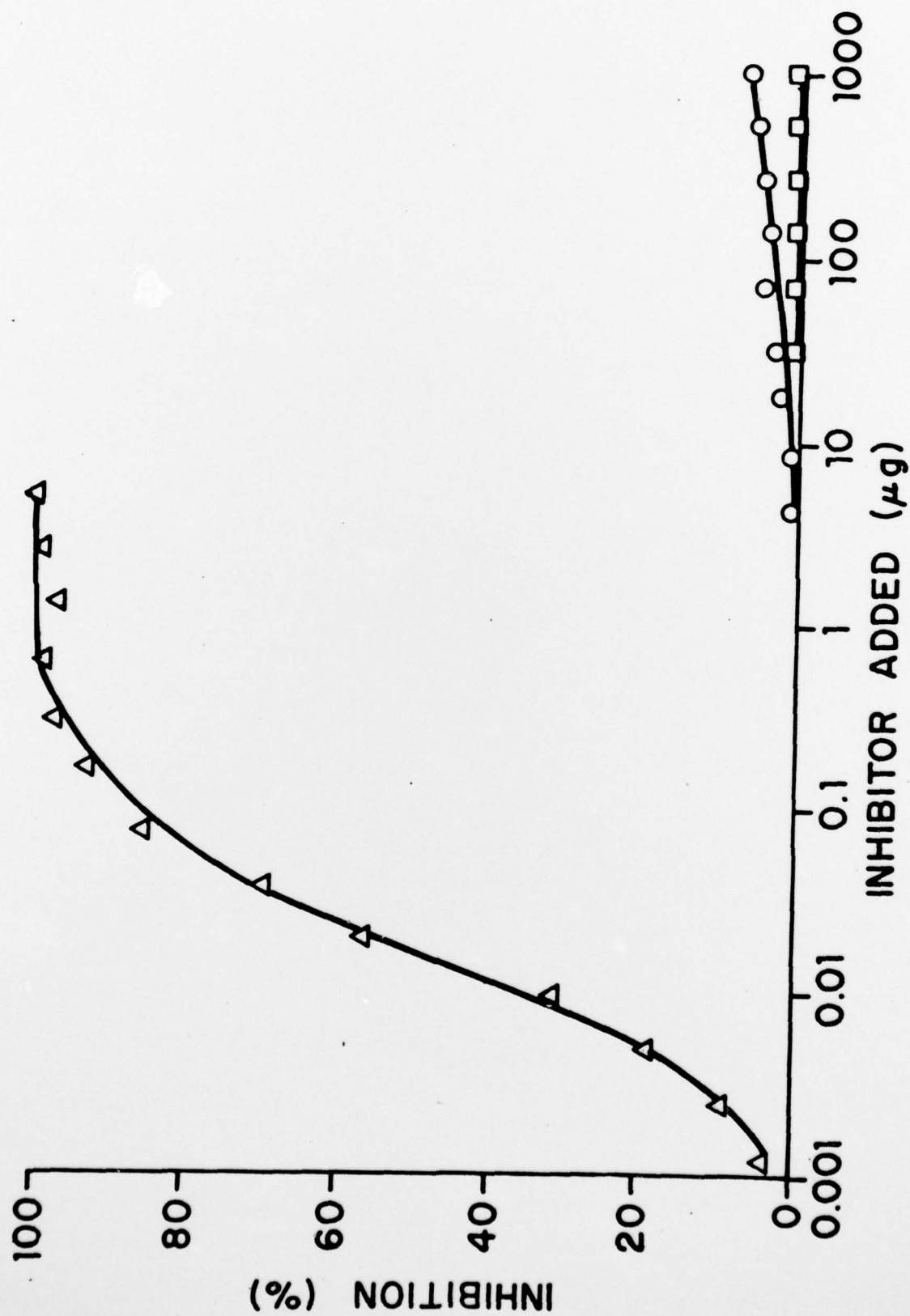


Fig. 5

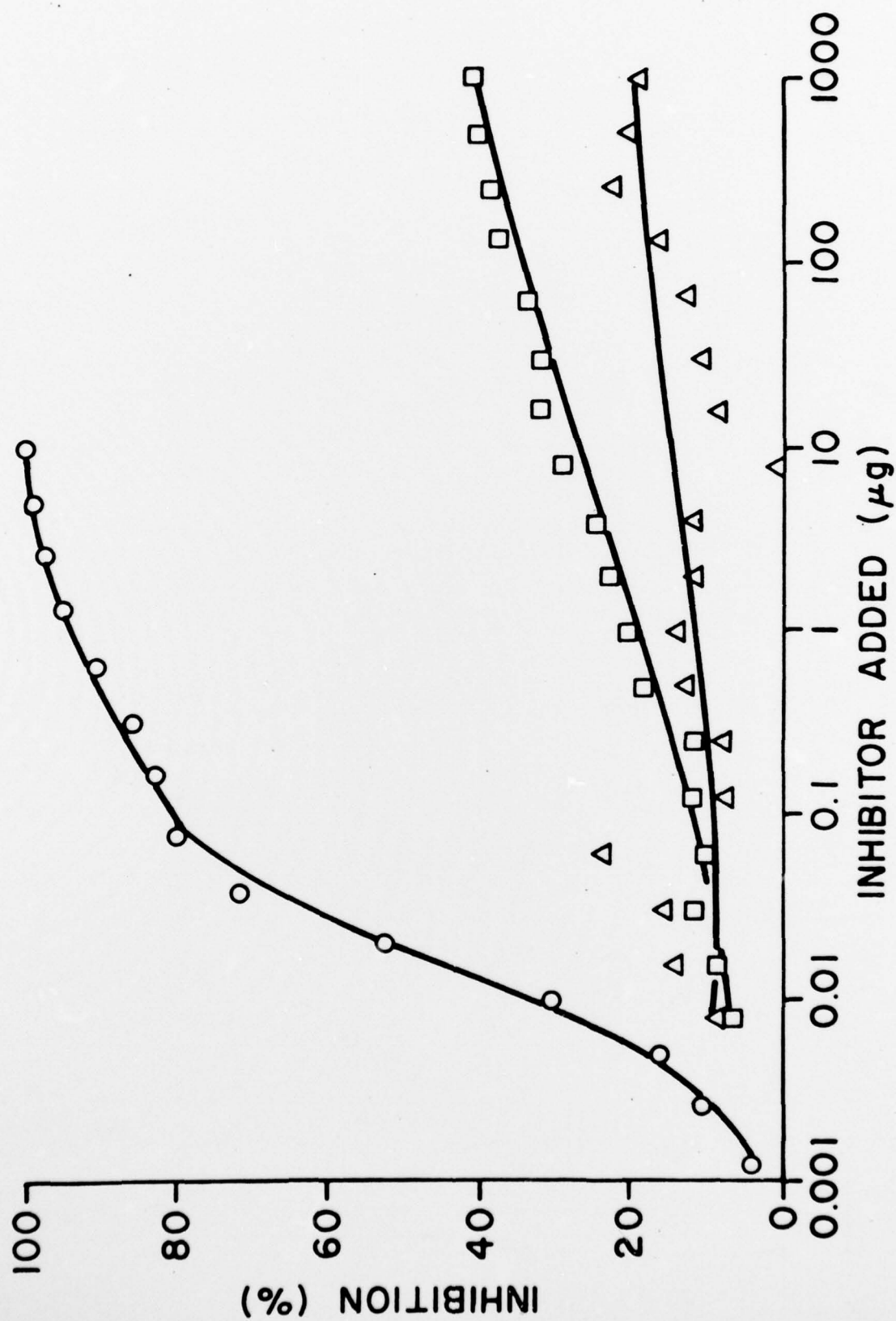


Fig. 6

